INDUSTRIAL METHOD FOR PRODUCING RNA AND SYSTEM FOR CARRYING OUT SAID METHOD

The present invention relates to an industrial method for producing RNA.

The present invention also relates to a system for carrying out the industrial production of RNA.

There exists in fact a need in the pharmaceutical industry for the production of large amounts of RNA for producing medicinal products (interfering RNA, essentially) and for research on RNAs(crystallization, NMR, complexes). There also exists a need for studying the effect of stopping expression of a given gene on cell (interfering RNA, etc…).

As regards, for example, methods normally used for producing RNA, it includes *in vitro* transcription and chemical synthesis reactions.

In vitro transcription reactions use bacteriophage (SP6, T7 or T3) RNA polymerases. The yield from these synthesis reactions and the amounts of product obtained is in general limited, in particular because of limiting factors such as the nucleotide concentrations (inhibitory effect of nucleotide concentrations > 8 mM, for example), the concentrations of the various elements constituting the reaction medium, and in particular the concentration of Mg^{++} ions. In general, it is accepted that magnesium concentrations must be in excess in *in vitro* transcription reactions. The use of pyrophosphatase, in combination with Mg^{++} ions, has also been proposed and is considered to improve the transcription reaction yield.

Another complication encountered in the *in vitro* synthesis of polynucleotides is the inhibition of the phage polymerases at relatively low ion concentrations.

In order to avoid these various drawbacks,

the use of improved reaction media has been proposed in US patent 5,256,555, which describes the use of a reaction medium comprising high total molar concentrations of nucleotides (between 12 mM and 40 mM) which were previously considered to be inhibitory concentrations, and an effective molar amount of Mg^{++}, which is below saturation with regard to the total molar concentrations of nucleotides, of pyrophosphatase and of Mg^{++}-nucleotides or tris-nucleotides.

Despite the various improvements proposed, *in vitro* transcription for producing RNAs has the following drawbacks:

- induction of parasitic reactions (N+1 activity) which increase the heterogeneity of the transcription products and require a thorough purification of the RNA;
- limitation with regard to the amount and to the size of the RNA synthesized;
- relatively high production costs.

The applicant consequently gave itself the aim of providing a method for producing RNA which does not have the drawbacks of the methods normally used and which thus more successfully meets the practical needs, in that it makes it possible to synthesize any RNA of interest in large amounts or with a high yield and at a cost that is significantly lower than that obtained with the *in vitro* methods of the prior art.

A subject of the present invention is a method for producing RNA molecules, which method is characterized in that it comprises at least the following steps:

(1) transforming yeast cells, in particular of *S. cerevisiae*, lacking mitochondrial DNA (*rho*^{0} cells) with a mitochondrial transcription vector comprising the DNA encoding the RNA of interest, regulatory elements for mitochondrial transcription, RNA maturation and stability, and a mitochondrial transformation reporter gene or a fragment of said reporter gene; the method according to the invention

thus allows the transcription of any DNA sequence whatever its intraspecific or interspecific origin (including DNAs of mitochondrial or chloroplastic origin, and also synthetic DNA sequences that do not exist naturally; a mitochondrial transformant or a synthetic *rho*$^-$ strain is thus obtained;

(2) identifying the yeast mitochondrial transformants that have incorporated the DNA of interest;

(3) culturing the yeast mitochondrial transformants selected in step (2), preferably until mid-exponential growth phase;

(4) isolating the mitochondria from the yeast mitochondrial transformants obtained according to step (3), and

(5) extracting and purifying the RNA of interest obtained from said mitochondria.

Under the conditions of the methods according to the invention, the RNA obtained is stable.

Definitions (see figures 2 to 4)

- *rho*$^+$ Strain: in these strains, the mitochondrial DNA is intact and functional; they include wild-type strains as well as mutant strains such as rho$^+$ mit$^-$ strains(figure 2).

- *rho*^{0} mutants: mutants which have lost their entire mitochondrial DNA leading to loss of respiratory growth but persistence of fermentative growth. Indeed, some mitochondrial genes encode essential subunits of the respiratory chain, and the rest for the protein translation system of mitochondria. So there are no more proteins encoded by the mitochondrial genome in a rho^0 strain(figure 2).

- *rho*$^-$ mutants:in this type of mutant mitochondrial DNA has undergone large deletions (> 50%) making it nonfunctional. Mitochondrial DNA is conserved repeated end-to-end to make a molecule of equivalent size to a molecule of *rho*$^+$ mitochondrial DNA. But in any *rho*$^-$ strain, the elements of the mitochondrial protein synthesis system are lost (as distributed

throughout the mitochondrial genome), but the remaining mitochondrial DNA can still be transcribed into RNA (only dependent on nuclear factors) (Figure 2). *Rho-* mutation thus leads to an absence of all proteins encoded by mitochondrial DNA, as a *rho*^{0} strain. The phenotype described as petite colony corresponds to both a *rho*$^-$ and a *rho*^{0}.

- Synthetic *rho*$^-$ strain:it is a strain originally *rho*^{0} in which DNA was artificially brought in into mitochondria (through a technique of bombardment (biolistic). It uses a property of yeast which is able to replicate and maintain any fragment of circular DNA in its mitochondria in the absence of mitochondrial DNA (a bacterial vector for example). The fragment introduced is then repeated end-to-end several times to reconstitute a DNA molecule of similar size to a molecule of *rho*$^+$ mitochrondrial DNA. In mitochondria of these cells, a sequence of any DNA inserted between sequences for mitochondrial transcription may actually be transcribed. By contrast, the translation will not occur because the necessary elements normally present in mitochondrial DNA, are absent here. It is for these properties similar to those of *rho*$^-$ cells (natural) that such cells were called by analogy synthetic *rho*$^-$ (Figure 4).

- mit$^-$ mutants: mutants that comprise a mutation in mitochondrial DNA that specifically affects the respiratory function (mutants located in one of several genes of mitochondrial subunits of respiratory complex) but that does not affect mitochondria protein translation function.

- Mitochondrial transformants: they are obtained directly after a bombardment of the cells (biolistic). If one bombs rho^0, the mitochondrial transformants are synthetic *rho*$^-$. Any vector can be used for bombing, but if you want to easily identify mitochondrial transformants, a mitochondrial marker gene (or part) is required.

- Mitochondrial recombinants: they are obtained by

homologous recombination after the synthetic *rho*$^-$ strain and the *rho*$^+$ strain are brought together.

- Auxotrophy marker: mutation in a known gene of the pathway for biosynthesis or use of an amino acid, of a nucleotide, of a carbon-based substrate, etc.

Surprisingly, the method according to the invention:

- can be readily industrialized (use of conventional fermenters), and

- effectively makes it possible to obtain RNA of interest in large amounts, for a low cost, after a purification that can be readily set up.

In addition, it has the following advantages:

- the fact that it includes an *in vivo* synthesis, in yeast, of the RNA of interest allows it to benefit from all the cellular quality controls, in particular: (1) high fidelity of transcription, considerably minimizing the risks of error of incorporation (by several orders of magnitude compared with existing methods),

- the production costs are essentially independent of the length of the RNA and of the amount of RNA produced. Indeed, once the RNA producing strain is made, the production costs consist essentially of the purchase of relatively inexpensive reagents (culture media and products for purifying the RNA), whereas the methods of the prior art use very expensive reagents (nucleotides, enzymatic kits) in large amounts.

Such a method therefore constitutes a particularly advantageous alternative to the methods for producing RNA *in vitro*, according to the prior art.

Indeed, yeast is a host of choices in the method according to the invention because there is no editing in mitochondria; consequently, the RNA molecules which are produced will not be modified post-transcriptionally even though they are produced in

vivo. In the prior art, mitochondrial genetic transformation in *S. cerevisiae* are designed to express protein markers, but also to introduce, in the mitochondrial DNA, mutations that were previously created *in vitro* on the corresponding cloned sequence; it is shown for the first time that yeast cells lacking mitochondrial DNA (rho^0 cells) can be used for industrial production of RNA of interest *in vivo*.

For example, the article by N. Bonnefoy et al. (Meth. Enzymol., 2001, 150, 97-111) summarizes the properties and characteristics of mitochondrial DNA from *S. cerevisiae* and in particular phenotypes associated with expression of mitochondrial genes, replication of mitochondrial DNA, recombination and segregation of mitochondrial DNA. In addition, this article studies yeast transformation process via their bombardment with exogenous DNA adsorbed on particles. More specifically, the system described in this article implements a sequence of interest which is mitochondrial DNA and which contains the marker gene.

Contrary to what is stated in this article, the invention uses synthetic rho^--cells constructed from rho^0 cells, able to produce a preselected RNA of interest. In the invention, first, the sequence of interest has generally no relationship with mitochondrial DNA, and secondly the marker is a mitochondrial sequence, for example, the *COX2* gene, which allows to identify mitochondrial transformants ("marker rescue") (Figure 3).

According to a first advantageous embodiment of the method according to the invention, prior to step (1), said DNA encoding the RNA of interest is amplified to be cloned into said mitochondrial transcription vector.

In accordance with the invention, the DNA encoding the RNA of interest can be amplified by PCR. In such a case, the oligonucleotide primers are established in the following way: the oligonucleotide P1 is complementary to the 5′ region of the DNA of interest, adjacent to transcription +1. It comprises a restriction site for cloning the amplified DNA into the transformation vector and, optionally, a site that facilitates purification of the RNA of interest. The restriction site can be cleavable and can be either on the plasmid on or the primer. The oligonucleotide P2 is, itself, complementary to the 3′ region of the DNA of interest, adjacent to the transcription stop. Here also, the oligonucleotide advantageously comprises a restriction site for cloning the amplified DNA and, optionally, sequences that allow a more or less extensive purification of the RNA of interest. The sequences are not necessarily in the oligonucleotide but can be found also on the plasmid.

According to another advantageous embodiment of said method, the regulatory elements for mitochondrial transcription, RNA maturation and stability included in the mitochondrial transformation vector are advantageously a transcription unit. Mention may be made, for example, of signal sequences for Cox2 and Cox1 expression. The signal sequences of other mitochondrial genes can also be used.

According to another advantageous embodiment of the method according to the invention, the mitochondrial transformation reporter gene is advantageously a gene encoding one of the proteins of the yeast respiratory chain [genes for apocytochrome b and for subunits I, II and III of cytochrome oxidase (*COX*)] or a mitochondrial gene for ATP synthase.

The vector used is preferably a vector of bacterial origin, for example pUC18, comprising a

mitochondrial transformation reporter gene or a fragment thereof (*COX2*, for example). According to the invention, when such vector is implemented, only two RNAs are produced in the system according to the invention: *COX2* and the RNA of interest. It is therefore easy to separate these two RNAs by virtue of their respective sizes, for example by electrophoresis, HPLC, NMR, affinity, etc. In addition, the use of a part only of the reporter gene implies that it is not transcribed and consequently makes the purification of the RNA of interest more easy which then finds itself as the one and only RNA in the mitochondria.

This vector can be improved in the following way: by introducing (i) sequences allowing higher production of the RNA of interest, for example by the addition of an Ori sequence (origin of replication of the mitochondrial DNA) of *S. cerevisiae*, so as to increase the efficiency of replication of the vector in the mitochondria, and/or (ii) sequences that facilitate purification of the RNA, and optionally sequences for removing the maturation sequences; to assay the mitochondrial transformation in the mitochondrial transformation vector, the gene encoding an element of the respiratory chain, and in particular the COX2 gene can be replaced with a region of said gene allowing complementation by homologous recombination of a mit^- allele of *COX2* present in the transformation tester strain. The RNA of interest is then the one and only RNA present in the mitochondria of the synthetic rho^- strain.

According to another advantageous embodiment of the method according to the invention, the transformation according to step (1) comprises the fixation of said mitochondrial transcription vector onto metal beads(tungsten or gold) and the projection of said beads onto said cells, in a manner known per se, in accordance, for example, with the biolistic method as described in the article by N. Bonnefoy et al., (*Methods in Enzymology*, 2001, 350, 97-111). The

device used is, for example, a PDS-1000/He system (BioRad). This instrument uses a helium shock wave to accelerate microscopic particles in the direction of a layer of cells on a Petri dish. The size of these particles is 0.45 µm in diameter, which represents approximately 10% of the size of a yeast cell. In a limited number of cells, these microprojectiles cross the yeast wall and reach the mitochondrion. Since the mitochondria of *rho*^{0} cells do not contain any of their own DNA, the DNA introduced by biolistics is the only DNA present in these organelles and synthetic *rho*$^-$ cells are thus obtained.

According to another advantageous embodiment of the method according to the invention, the yeast cells lacking mitochondrial DNA are *rho*^{0} strains or modified *rho*^{0} strains. By way of example of modified *rho*^{0} strains, mention may be made of the following strains: the *rho*^{0} strains derived from the DBY947 strain: ATCC 201440 (MCC109*rho*^{0} [MATa, ade2-101, ura3-52, karl-1 (*rho*^{0})]) ATCC 201442 (MCC123*rho*^{0} [MATa, ade2-101, ura3-52, karl-1 (*rho*^{0})]) and DFS160*rho*^{0} (M.E. Sanchirico et al., EMBO J., 1998, 17, 19, 5796-5804. d; Steele et al., PNAS, 1996, 93, 5253-5257).

Said yeast strains cells can be advantageously modified such that the genes encoding some RNA degradation proteins in the mitochondrion are modified or eliminated. Several mitochondrial proteins, all of nuclear origin, that are involved in the turnover of mitochondrial RNAs (for example Suv3p, a subunit of a 3'-5' exoribonuclease) are known to date. By using yeast strains in which the genes of these proteins have been eliminated (these strains are available), it is possible to increase artificially the stability of the RNAs synthesized in the mitochondrion by the method of the invention.

The majority of mitochondrial proteins are of nuclear origin. This is in particular the case for the protein machinery necessary for the transcription of DNA to RNA. This machinery is therefore imported into

the mitochondrion and remains functional even when the cells are *rho*^{0} and therefore lacking mitochondrial DNA. On the other hand, part of the machinery for the translation of RNA to protein is encoded by the mitochondrial genome. Consequently, if the mitochondrial genome is absent - this is the case in the synthetic *rho*$^-$ cells, the foreign DNA introduced into the mitochondrion is not translated to protein. In this context, the DNA introduced by biolistics is the only DNA present in the mitochondria of the synthetic *rho*$^-$ cells obtained. It will be transcribed to RNA, but this RNA will not be translated to protein.

According to another advantageous embodiment of said method, step (1) comprises cotransforming the yeast with said mitochondrial transcription vector and a vector that is replicative in yeast, comprising a nuclear selection marker, for example, pFL46L (ATCC No. 77210) or Yep351 (ATCC No. 37672) (figure 4).

Said nuclear marker complements advantageously an auxotrophy marker for the transformed strain. In fact, it is the wild-type gene carried by the plasmid which functionally complements the mutated gene carried by the transformed strain.

According to another advantageous embodiment of the method according to the invention, step (2) comprises:

(a_0) crossing the synthetic *rho*$^-$ transformed yeast, obtained at the end of step (1), with a yeast tester strain of *rho*$^+$ mit$^-$ type, so as to facilitate the identification of said transformed cells, and in which a point mutation is present in a region corresponding to the mitochondrial transformation reporter gene used in step (1), for example one of the genes of the yeast respiratory chain (for example *COX2*) and whose corresponding wild-type sequence is carried by the mitochondrial transcription vector used to transform the mitochondrion of the *rho*^{0} host strain in step (1),

(b_0) identifying the mitochondrial transformants (synthetic *rho*$^-$ cells) that, once

crossed, give diploid cells capable of growing on a non-fermentable medium: only the cells of the host strain that contain, in their mitochondria, the mitochondrial transcription vector carrying the gene of interest and the wild-type allele of the mit$^-$ mutation present in the mitochondrial DNA of the tester strain will give, after recombination of the parental mitochondrial DNAs, *rho*$^+$ mit$^+$ recombinant diploid cells which will be revealed by their ability to grow on a non-fermentable medium, after replica-plating of the diploids on velvet, on such a medium. On this medium, for example, when the marker gene is incomplete (partial sequence), neither the parents of the cross (*rho*^{0} host strain and *rho*$^+$ mit$^-$ tester strain), nor the *rho*$^+$ mit$^-$ nonrecombinant diploids derived from this cross, will be capable of growing. This step makes it possible to define, on the initial dish obtained after bombardment of the *rho*^{0} host strain, areas where there are cells of this host strain in which the mitochondria have acquired the mitochondrial transcription vector carrying the gene of interest, and

(c_0) repeating said crossing until isolated yeast colonies identified as being mitochondrial transformants carrying the mitochondrial transformation vector (synthetic *rho*$^-$ cells) are obtained.

More specifically, the nuclear transformants obtained after bombardment are crossed with a strain which itself has a *rho*$^+$ mitochondrial DNA but also a mit$^-$ mutation which prevents it from growing on a respiratory medium, and which is covered by the marker gene (whole or in part) present in the synthetic *rho*$^-$. In this way, after crossing, the mutated mitochondrial DNA and the corresponding wild-type sequence on the synthetic *rho*$^-$ will be present together. Homologous recombination will then make it possible to correct the mit$^-$ mutation, and the diploids obtained will again possess respiratory growth, hence the term "marker rescue". In practice: there are considered to be 1000 to 10 000 nuclear transformants distributed over the

dish after bombardment, among which it is intended to identify those which are also mitochondrial transformants. This dish is therefore replica-plated on velvet (to keep the same arrangement of the clones on the Petri dish) on a dish of the same medium (stock dish) and on a layer of the *rho*$^+$ mit$^-$ tester strain. After crossing, the latter is replica-plated on a respiratory medium in order to pinpoint the clones which have allowed the marker rescue. By then going back to the stock dish (not crossed, replica of the original dish), the corresponding haploid synthetic *rho*$^-$ clones are recovered since these are the ones that are ultimately selected (figure 4).

In other words, the two strains that are crossed in step (a_0) cannot grow on a non-fermentable medium. It will therefore be easy to pinpoint the recombinant clones that have recovered the ability to grow on this type of medium. It is not therefore necessary to select the diploids on a specific medium (specific in terms of auxotrophic markers) before replica-plating the crosses on a non-fermentable medium. Only the cells of the host strain that contain, in their mitochondria, the mitochondrial transcription vector will be able to give, after crossing, clones that can grow on a non-fermentable medium.

Several cases are possible: recombinant diploids, but also nonrecombinant diploids if the marker gene is whole in the vector (trans-complementation by the RNA produced by the DNA of the synthetic *rho*$^-$), and, finally, recombinant or non-recombinant cytoductants (the kar 1-1 mutation of one of the two strains delays fusion of the nuclei and makes it possible to obtain, after crossing, haploids which have nevertheless undergone fusion of the cytoplasms and therefore of the mitochondria).

In step (c_0), the purification of the mitochondrial transformants is carried out by taking from the stock dish (not crossed) the area in which a clone has been pinpointed which gives, after crossing, growth

on a non-fermentable medium, and repeating the crossing after re-plating of the cells as individual colonies; this step (c_0) therefore makes it possible to select, at the second or third round, a colony of a pure mitochondrial transformant. In fact, the problem is that, among 5000 clones on a single dish, it is not possible to be sure to have recovered a pure clone, but only a mixed area from which it is preferable to purify the synthetic *rho*$^-$. For this, the recovered area is plated as individual colonies and the same tester cross is re-performed. After approximately 3 rounds of such purification, a pure synthetic *rho*$^-$ clone is obtained.

As a variant, step (2) comprises:

(a_1) a first selection or preselection of the yeast cells by means of the nuclear marker, by culturing in a suitable medium,

(b_1) a second selection from the yeast cells selected in (a_1), in accordance with steps (a_0), (b_0) and (c_0), as defined above.

For example, once the *rho*^{0} cells have been bombarded, they are incubated at 28°C, the optimal temperature for yeast growth. Firstly, the cells are incubated on a selected medium lacking the amino acid or the nucleotide corresponding to the auxotrophy marker of said cells. This mutation makes the growth of said cells dependent on the addition to the culture medium, for example, of the amino acid that can no longer be synthesized. To identify the mitochondrial transformants, the yeast capable of growing on selected medium are crossed with a strain having an adequate sex sign. This strain is mit$^-$, its mitochondrial DNA is present, but the COX2 gene has been deleted. The diploids are selected on a medium with a non-fermentable carbon source. Only the yeast in which the mitochondria have been transformed with the plasmid carrying *COX2* can form viable diploids with the strain mit$^-$ cox2- on this type of medium (complementation by recombination or translation of the *COX2* "wild-type" RNA. This cross is then repeated until isolated

colonies identified as being mitochondrial transformants carrying *COX2* are obtained (figure 4).

Preferably, and in accordance with the invention, after crossing with a strain carrying a mit^- mutation in the *COX2* gene, the complementation of this mutation can take place transiently in trans by translation of the messenger RNA of the gene *COX2* provided by the synthetic rho^- strain, after fusion of the parental mitochondrial networks. This crossing constitutes a test for identifying and selecting the transformants of interest with a view to producing the RNA of interest.

In other words, after the bombardment of the rho^0 cells, the nuclear transformants are first of all preferably selected. There is a large number of them (5000 per Petri dish), such that the colonies which they produce are not well separated from one another. Therefore, in a first step, the crosses with the tester strain (by replica-plating on velvet) will make it possible to define, on the original shot dish, regions containing mitochondrial transformants (in general about ten per dish). The cells of this area will be diluted and re-plated on a solid medium, so as this time to obtain well-separated colonies. These colonies are again tested by crossing with the mit^- tester strain so as to identify those derived from cells containing, in their mitochondria, the DNA of interest. Eventually, this step will be repeated again if necessary for finally obtaining the transformant of interest in a pure clonal form.

According to another embodiment of the method according to the invention, the isolation of the mitochondria, in accordance with step (4) of the method according to the invention, advantageously comprises, after lysis or grinding of said cells, isolation of the mitochondria according to methods known *per se* (Guérin B. et al., *Methods Enzymol.*, 1979, **55**, 149-59) or on a sucrose gradient.

Preferably, step (4) of the method according

to the invention advantageously comprises, after lysis or grinding of said cells, isolation of the mitochondria by means of at least two appropriate centrifugation steps, preferably at speeds of between 750 g and 12 500 g, and recovery of the final centrifugation pellet.

In accordance with the invention, the purification of the RNA according to step (5) can be carried out by techniques known *per se* (di Rago JP. et al., *J. Biol. Chem.*, 1988, **263**, 12564-12570).

However, the purification of the RNA according to step (5) comprises advantageously:

- lysing the final centrifugation pellet containing mitochondria obtained in step (4), in the presence of at least one detergent, a divalent ion-chelating agent, and within a pH range of between 7 and 8; by way of example, mention may be made of the following buffer: 1% SDS, 10 mM EDTA and Tris/HCl, pH 7.5;
- eliminating the contaminating nucleic acids, in particular numerous RNAs attached at the periphery of the mitochondrion, in the presence of suitable buffers comprising at least one divalent ion-chelating agent; such buffers include buffers comprising EDTA and EGTA;
- eliminating the cytoplasmic RNAs situated in the periphery of the mitochondria and the remaining nuclear DNA by incubating the mitochondria in a suitable buffer lacking divalent ion-chelating agent and in the presence of RNase and DNase,

and

- isolating and purifying the nucleic acids by successive phenolic extractions.

Advantageously, the RNA thus purified is quantified, analyzed on an agarose gel and sequenced.

Also, said RNA can be used advantageously as it is or can be subjected to additional purification steps to meet the demands (cleavage of the maturation sequences; chemical modification, double-stranded

hybridization, digestion so as to obtain small fragments covering the entire gene, etc..).

A subject of the present invention is also the use of synthetic rho$^-$ yeast cells lacking mitochondrial DNA as defined above, for the industrial production of a preselected RNA of interest.

A subject of the present invention is also a system for carrying out the industrial production of a preselected RNA of interest, characterized in that it comprises:

- yeast cells transformed at least with a mitochondrial transcription vector (synthetic *rho*$^-$ cells) comprising the DNA encoding the RNA of interest, regulatory elements for mitochondrial transcription, RNA maturation and stability, and a mitochondrial transformation reporter gene or a fragment of said reporter gene,
- at least one suitable culture medium for selecting said transformed cells (mitochondrial transformants),
- tester yeast cells of *rho*$^+$ mit$^-$ type,
- appropriate fermenters and culture media, and
- appropriate buffers for isolating the mitochondria from the synthetic *rho*$^-$ cells and extracting the RNA of interest therefrom.

Besides the above arrangements, the invention also comprises other arrangements that will emerge from the description which follows, which refers to examples of implementation of the method that is the subject of the present invention and also to the attached drawings in which:

- figure 1 illustrates the results of Northern blotting.
- figure 2 illustrates the mitochondrial DNA of *S. cerevisiae*; the mitochondrial DNA encodes in particular: 3 subunits of ATP synthase (6, 8, 9); 4 subunits of the respiratory chain; 2 rRNAs + 1 mito-ribosome; 24 tRNAs;

- figure 3 illustrates the principle of marker rescue;

- figure 4 represents the construction of a synthetic *rho*$^-$ strain;

It should be clearly understood, however, that these examples are given only by way of illustration of the subject of the invention, of which they in no way constitute a limitation.

EXAMPLE 1: Materials and methods

1) Mitochondrial transcription vector

pJM2 (pTZ18-U, with wild-type *COX2*; YIN J. et al., *Tsinghua Science and Technology*, 1999, **4**, 2; Bio-Rad) into which the *RIP1*^{m} gene has been cloned, mitochondrial-code version of the *RIP1* gene encoding a subunit of respiratory chain complex III, flanked by the *COX1* expression signal sequences.

2) Shuttle vector comprising a nuclear selection marker

Yep352 (2 µ, URA3) (ATCC No. 37673.)

3) Transformation of *S. cerevisiae* cells by microprojectile bombardment (Biolistic PDS-1000/He with the vectors as described in 1) and 2)

W303-1B (ATCC No. 201238)/A/50: *rho*^{0} derivative of W303-1B (Matα, ade2, trp1, his3, leu2, ura3).

4) Method for identifying the transformed cells

Firstly, the nuclear transformants are selected on a synthetic medium lacking uracil. The mitochondrial transformants are identified among the URA3$^+$ nuclear transformants by virtue of their ability to produce diploids with growth on non-fermentable medium after crossing with the non-respiring tester strain TF145 (*MATα, ade2*) (Speno H. et al., *J. Biol. Chem.*, 1995, **270**, 43, 25363-25369) which carries a deletion mutation of *COX2* (*cox2-17*) in the mitochondrial DNA.

5) Yeast cell culture conditions:

The mitochondrial transformants are cultured in fermenters until an exponential growth phase medium is obtained, in a rich medium containing galactose as

carbon source.

6) Method for purifying the mitochondria and the RNA:

6.1) Isolation of mitochondria

The yeast mitochondrial transformants are cultured in a fermenter until an exponential growth phase medium is obtained, i.e. an OD of between 3 and 4. They are then harvested and lysed or ground and their mitochondria are purified by conventional methods.

Briefly, the protocol used is as follows:

The mitochondria of the mitochondrial transformants are isolated and purified after digestion of their cell wall with zymolyase in a medium (1.35 M sorbitol) that osmotically protects the integrity of the cells that have had their wall removed (called spheroplasts). The spheroplasts are subjected to an osmotic shock in a buffer that preserves the integrity of the mitochondria (0.6 M sorbitol). The cell debris (nuclei, walls) are removed by several (a minimum of 2) low-speed (750 g) centrifugations of the spheroplast lysate. The final supernatant is centrifuged at high speed (12 500 g) so as to pellet the mitochondria.

More specifically, the conditions are as follows:

I/ Harvesting of yeast

The yeast are centrifuged at low speed (4°C, 10 minutes at 3800 g).

II/2 Washing of the yeast with ice-cold distilled water

The pellets are then taken up in chilled distilled water (4°C).

Centrifugation is carried out at 4°C for 5 min at 3800 g. The supernatant is removed and washing is carried out a second time with distilled water, followed by a further centrifugation under the same conditions.

III/ Weakening of the cell wall

2-Mercaptoethanol breaks the disulfide bridges between the various mannoproteins of the wall,

facilitating the subsequent action of the zymolyase. Based on the OD 600 nm of the culture, the dry weight of yeast is evaluated by means of the following formula:

DW (g) = 0.28 OD volume_culture (L)

Incubation is carried out in a volume of 20 ml of buffer/g of DW.

The pellet is therefore taken up in a pre-incubation buffer "SH" (0.5 M 2-mercaptoethanol, 0.1 M Tris-Base, pH 9.3) and is incubated for 10 min at 30°C with agitation.

IV/ Washing of the yeast with the KCl washing buffer

The aim of these washes is to remove all traces of reducing agent.

The KCl buffer (0.5 M KCl, 10 mM Tris-Base, pH 7.0) is added to the SH preincubation buffer. Centrifugation is carried out at 4°C for 5 min at 3800 g. The supernatant is removed. Two successive washes with the KCl buffer are thus carried out.

V/ Digestion of the cell wall with zymolyase at 30°C

Zymolyase destroys the cell wall.

The yeast wall consists of a chitin backbone to which other proteins are added.

In order to digest the wall, one possibility is to use the zymolyase produced by the bacterium *Arthrobacter luteus* or an enzymatic mixture (cyto-helicase): snail (*Helix pomatia*) gastric juice enzyme, thereby giving yeast protoplasts.

The pellet is taken up with the digestion solution, in a proportion of 10 to 15 mg of zymolyase/ 10 ml of digestion buffer (1.35 M sorbitol, 1 mM EGTA, 10 mM citric acid, 30 mM disodium phosphate, pH 5.8).

The zymolyase digestion is stopped when 80 to 90% of the cells are digested, by making up the volume with the buffer for washing the protoplasts with KCl.

Centrifugation is then carried out for 5 min at 12 500 g.

VI/ Washing of protoplasts

The pellet is taken up rapidly in the washing buffer for protoplasts (or spheroplasts = cells from which the wall has been removed) (0.75 M sorbitol, 0.4 M mannitol, 0.1% BSA, 10 mM tris-maleate, pH 6.8). Centrifugation is carried out for 5 min at 12 500 g. The supernatant is removed and then the pellet is washed a second time.

VII/ Homogenization and grinding

The pellets are taken up in a few ml of the homogenization buffer (0.6 M mannitol, 2 mM EGTA, 0.2% BSA, 10 mM tris-maleate, pH 6.8). The preparation is poured into a potter homogenizer. The potter homogenizer is moved up and down about ten times; the preparation is mixed at moderate speed, a maximum amount of the preparation is recovered, and the entire mixture is then redistributed into several tubes.

VIII/ Isolation of mitochondria by differential centrifugation

Low-speed (750 g) centrifugation is carried out for 8 min at 4°C. The supernatants are conserved. The pellet can optionally be taken up in the homogenization buffer and centrifuged and the supernatant added to the previous supernatant.

Centrifugation at higher speed (12 500 g) is carried out for 10 min at 4°C, and the supernatants are then removed.

The pellets are taken up in the recovery buffer (0.6 M mannitol, 2 mM EGTA, 10 mM tris-maleate, pH 6.8).

Low-speed centrifugation (8 min, 750 g at 4°C) is carried out, followed by high-speed centrifugation of the supernatants (10 min, 12 500 g, at 4°C). The supernatant is removed and a third low-speed/high-speed centrifugation cycle is optionally carried out.

IX/ Final recovery of mitochondria

The mitochondrial pellet is taken up in a minimum volume of recovery buffer (mannitol, EGTA,

tris-maleate, pH 6.8, as defined above), and then transferred into a potter homogenizer in order to be homogenized.

X/ Yield

Approximately 2-4 g of yeast are produced per 2 liters of culture.

A preparation of mitochondria with zymolyase makes it possible to obtain 1 to 1.5 ml of mitochondria at a concentration of 30 mg/ml of mitochondrial proteins, i.e. 30 to 45 mg of proteins.

RNA extraction

The mitochondria are, firstly, incubated in the presence of EDTA and of EGTA in order to remove from them the polysomes and the RNAs located at the periphery of the mitochondrion. They are then washed in buffer free of EDTA and of EGTA and incubated in this same buffer in the presence of RNase and of DNase in order to remove from them the cytoplasmic RNAs located at the periphery of the mitochondria and the remaining nuclear DNA. The action of the RNase and the DNase is interrupted by centrifugation at 12 500 g. The nucleic acids are then extracted as described by Di Rago et al., 1990. The mitochondrial pellet is washed in buffer containing EDTA and EGTA, and lysed in the presence of 1% SDS, 10 mM EDTA and Tris HCl, pH 7.5. The nucleic acids are purified by successive phenolic extractions. The final aqueous phase is optionally washed by the action of chloroform::isoamyl acid, and the nucleic acids are then precipitated. In this step, the mitochondrial DNA is optionally degraded by adding DNase. The RNA thus purified is assayed, analyzed on an agarose gel and sequenced.

More specifically, the mitochondrial RNA extraction protocol is as follows:

10 mM EDTA was added to the recovery buffer (defined above); the various centrifugations are carried out at 4°C.

- Centrifugation at 12 500 g for 10 min.
- Recovery in recovery buffer, without EDTA

or EGTA.

- Centrifugation at 12 500 g for 10 min.

- Pellet taken up in recovery buffer without EDTA or EGTA. Addition of RNase and of DNase and incubation at 37°C for 15 min.

- Centrifugation at 12 500 g for 10 min.

- Washes in recovery buffer with EDTA and EGTA, followed by centrifugations.

- Pellet taken up in lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris HCl, pH 7.5) and addition of the same volume of 49:49:2 phenol:chloroform:isoamyl alcohol mixture. Mixture vortex for 3 min, left to stand for 2 min at 4°C, revortex for 2-3 min.

- Centrifugation for 5 min at 8 000 g and at 4°C.

- Removal of the aqueous phase and addition of the phenol/chloroform mixture, approximately 5 times.

- Addition to the final aqueous phase of 0.3 M of sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol. Mixture left to stand for 15 min at -80°C and then centrifuged for 20 min at 8 000 g and at 4°C, and pellet washed.

- Pellet taken up in 3 ml of 10 mM $MgCl_2$, 20 mM Tris/acetate buffer, pH 7.4, containing 75 µl of 200 mM vanadyl-ribonucleoside complex (RNase inhibitor) and 2 µl of RNase-free DNase (in 50% glycerol at a concentration of 0.5 mg/ml).

- Removal of the DNase with one volume of 49:49:2 phenol/chloroform/isoamyl alcohol, and then one volume of 24:1 chloroform/isoamyl alcohol, and then one volume of water-saturated diethyl ether.

- Precipitation of the RNAs in the aqueous phase by addition of 0.3 M sodium acetate and 2.5 volumes of 100% ethanol.

- Pellet taken up in 50 to 100 µl of sterile water.

- Assaying and verification of the RNAs and then additional preparation and purification if

necessary.

EXAMPLE 2: Results

The RNAs thus obtained are analyzed by the Northern blotting technique with a P^{32}-labeled specific probe (RIP1 gene DNA). Figure 1 shows the results of the Northern-Blotting analysis whose protocol is described above. It appears that the mitochondrial RNAS extracted from a rho^{+} mit^{+} wild-type yeast strain (normal mitochondria) reacts normally with a probe specific for the endogenous mitochondrial gene cox1 and, also, those of a synthetic rho^{-} strain do not react with this probe. The analysis of the RNAs with a probe specific for the RIP1 gene did not give any signal with the wild-type control. By contrast, a signal of the expected size was detected with the mitochondrial RNAs of the synthetic rho^{-} strain.

As emerges from the above, the invention is in no way limited to those of its methods of implementation, execution and application that have just been more specifically described; on the contrary, it encompasses all the variants thereof that may occur to a person skilled in the art, without departing from the context or the scope of the present invention.

CLAIMS

1. A method for producing RNA molecules, which method is characterized in that it comprises at least the following steps:

(1) transforming rho^0 yeast cells lacking mitochondrial DNA with a mitochondrial transcription vector comprising at least one copy of the DNA encoding the RNA of interest, regulatory elements for mitochondrial transcription, RNA maturation and stability, and a mitochondrial transformation reporter gene or a fragment of said reporter gene, for producing synthetic rho^- cells or mitochondrial transformants;

(2) identifying the yeast mitochondrial transformants that have incorporated the DNA of interest;

(3) culturing the yeast mitochondrial transformants selected in step (2);

(4) isolating the mitochondria from the yeast mitochondrial transformants obtained in step (3), and

(5) extracting and purifying the RNA of interest obtained from said mitochondria.

2. The method as claimed in claim 1, characterized in that prior to step (1), said DNA encoding the RNA of interest is amplified to be cloned in said mitochondrial transcription vector.

3. The method as claimed in claim 1 or claim 2, characterized in that the elements for mitochondrial transcription, RNA maturation and stability contained in the mitochondrial transcription vector are advantageously a transcription unit comprising a promoter for transcription and a suitable terminator.

4. The method as claimed in any one of claims 1 to 3, characterized in that the mitochondrial transformation reporter gene is advantageously a gene encoding one of the proteins of respiratory chain or a mitochondrial gene for ATP synthase.

5. The method as claimed in any one of claims 1 to 4, characterized in that the transformation according to step (1) comprises the fixation of said

mitochondrial transcription vector onto metal beads and the projection of said beads onto said cells.

6. The method as claimed in any one of claims 1 to 5, characterized in that the yeast cells lacking mitochondrial DNA are advantageously *rho*^{0} strains, eventually modified.

7. The method as claimed in any one of claims 1 to 6, characterized in that step (1) comprises the cotransformation of the yeast with said mitochondrial transcription vector and a vector that is replicative in yeast, comprising a nuclear selection marker.

8. The method as claimed in claim 7, characterized in that said nuclear marker is an auxotrophic marker of said transformed strain.

19. The method as claimed in any one of claims 1 to 8, characterized in that step (2) comprises:

(a_0) crossing the yeast mitochondrial transformants obtained in step (1) with a yeast tester strain of *rho*$^+$ mit$^-$ type,

(b_0) identifying the mitochondrial transformants which, once crossed, give diploid cells capable of growing on a non-fermentable medium, and

(c_0) repeating said crossing until isolated yeast colonies identified as being mitochondrial transformants carrying the mitochondrial transformation vector are obtained.

10. The method as any one of claims 1 to 8, characterized in that step (2) comprises:

(a_1) a first selection or preselection of the yeast cells by means of the nuclear marker, by culturing in a suitable medium,

(b_1) a second selection from the yeast cells selected in (a_1), in accordance with steps (a_0), (b_0) and (c_0), as defined in claim 9.

11. The method as claimed in any one of claims 1 to 10, characterized in that the isolation of the mitochondria, in accordance with step (4) of the

method, comprises advantageously after lysis or grinding of said cells, the isolation of the mitochondria by at least two centrifugation steps, at speeds preferably of between 750 g and 12 500 g, and recovery of the final centrifugation pellet.

12. The method as claimed in any one of claims 1 to 11, characterized in that step (5) advantageously comprises:

- lysing the final centrifugation pellet obtained in step (4) containing the mitochondria, in the presence of at least one detergent, a divalent ion-chelating agent and within a pH range of between 7 and 8,

- eliminating the contaminating nucleic acids, in particular numerous RNAs attached to the mitochondrion periphery, in the presence of suitable buffers, comprising at least one divalent ion-chelating agent,

- eliminating the cytoplasmic RNAs located in the mitochondria periphery,and the remaining nuclear DNA by incubating the mitochondria in a suitable buffer lacking divalent ion-chelating agent and in the presence of RNase and DNase,

and

- isolating and purifying the nucleic acids by successive phenolic extractions.

13. Use of synthetic rho$^-$ yeast cells for the industrial production of a preselected RNA of interest.

14. A system useful for the industrial production of RNA of interest, characterized in that it comprises:

- synthetic rho$^-$ yeast cells transformed with at least one mitochondrial transcription vector as defined in claim 1,

- at least one suitable culture medium allowing the selection of said transformed cells,

- yeast tester cells of *rho*$^+$ mit$^-$ type,

- appropriate fermenters and culture media,

and

- appropriate buffers for isolating the mitochondria from synthetic *rho*$^-$ cells and extracting the RNA of interest therefrom.

ABSTRACT

Industrial method for producing RNA and system useful for said production. Said method of production of RNA molecules comprises at lesatthe following steps: (1) transforming rho^0 yeast cells lacking mitochondrial DNA with a mitochondrial transcription vector comprising at least one copy of the DNA encoding the RNA of interest, regulatory elements for mitochondrial transcription, RNA maturation and stability, and a mitochondrial transformation reporter gene or a fragment of said reporter gene, for producing synthetic rho^- cells or mitochondrial transformants; (2) identifying the yeast mitochondrial transformants that have incorporated the DNA of interest;(3) culturing the yeast mitochondrial transformants selected in step (2);4) isolating the mitochondria from the yeast mitochondrial transformants obtained in step (3), and(5) extracting and purifying the RNA of interest obtained from said mitochondria.

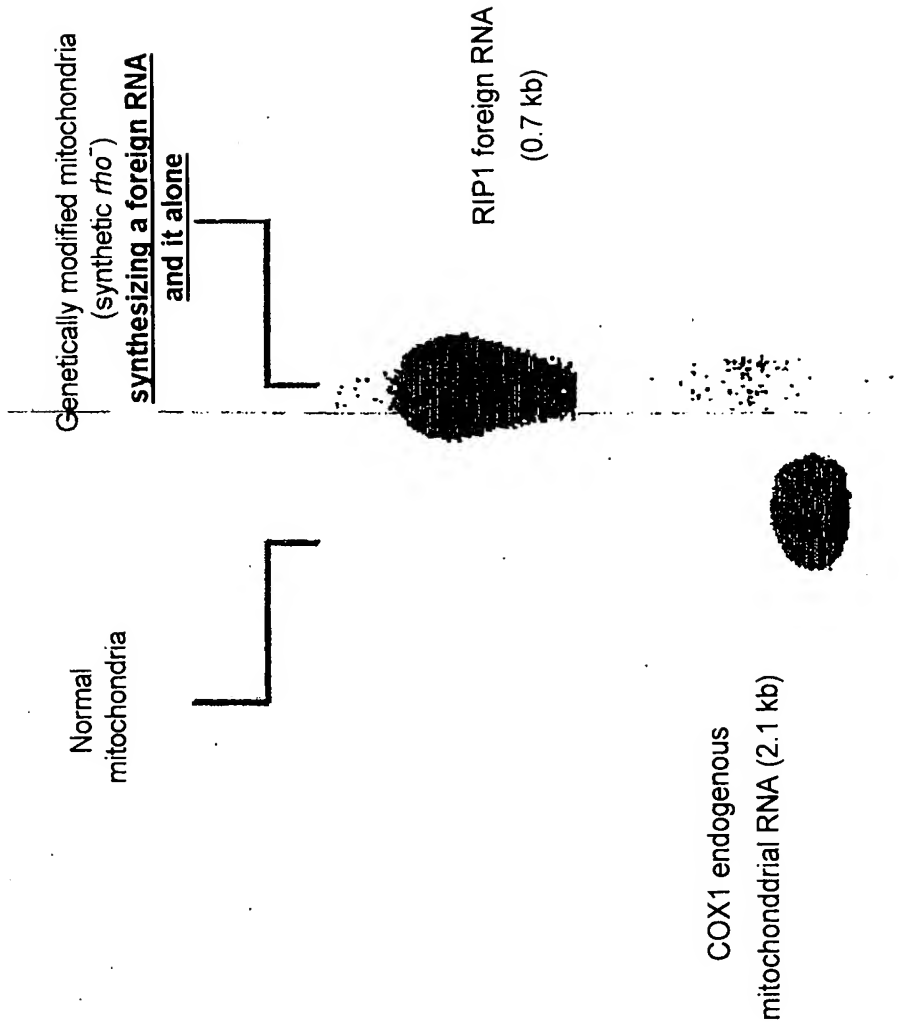
Genetically modified mitochondria
(synthetic *rho*⁻)
synthesizing a foreign RNA
and it alone
RIP1 foreign RNA
(0.7 kb)
Normal
mitochondria
COX1 endogenous
mitochonddrial RNA (2.1 kb)

FIGURE 1

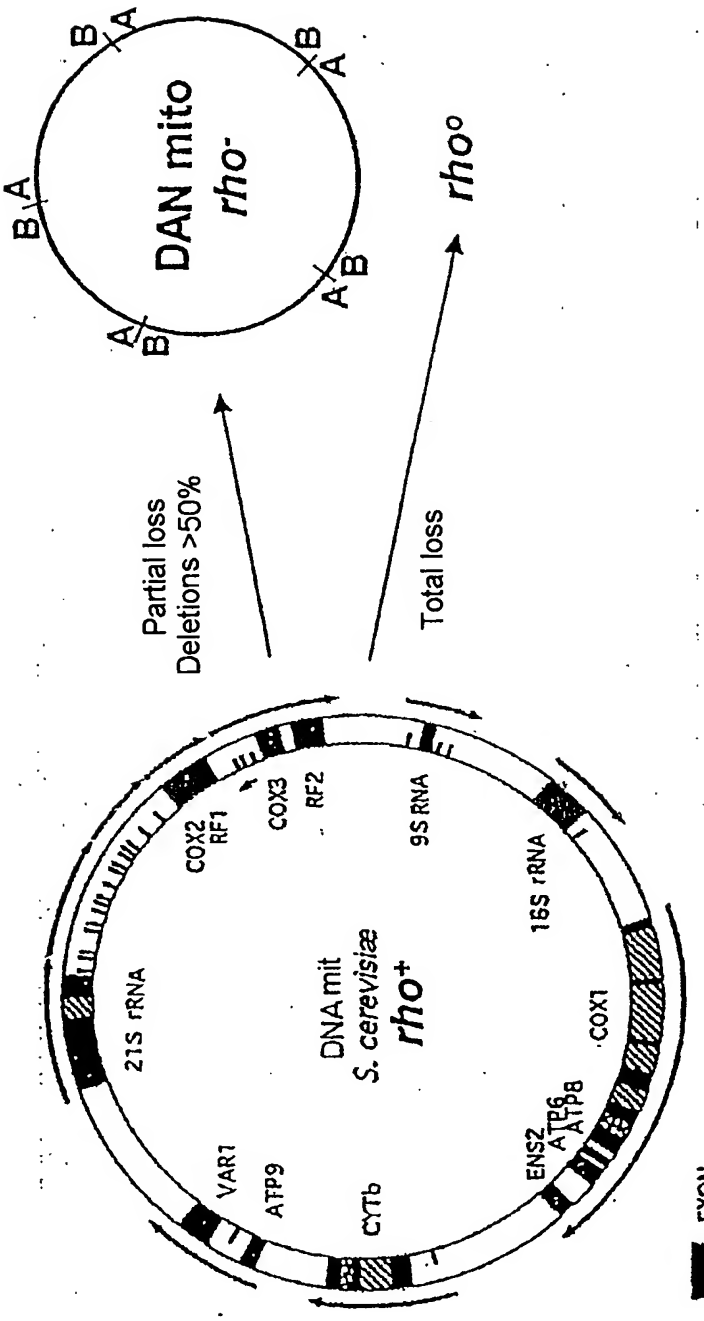
DAN mito
rho-
Partial loss
Deletions >50%
Total loss
rho°
DNA mit
S. cerevisiæ
rho+
21S rRNA
VAR1
ATP9
CYTb
COX2
RF1
COX3
RF2
9S RNA
16S rRNA
COX1
ENS2
ATP6
ATP8
EXON
CODING INTRON
NONCODING INTRON
tRNA
Transcription Unit

FIGURE 2

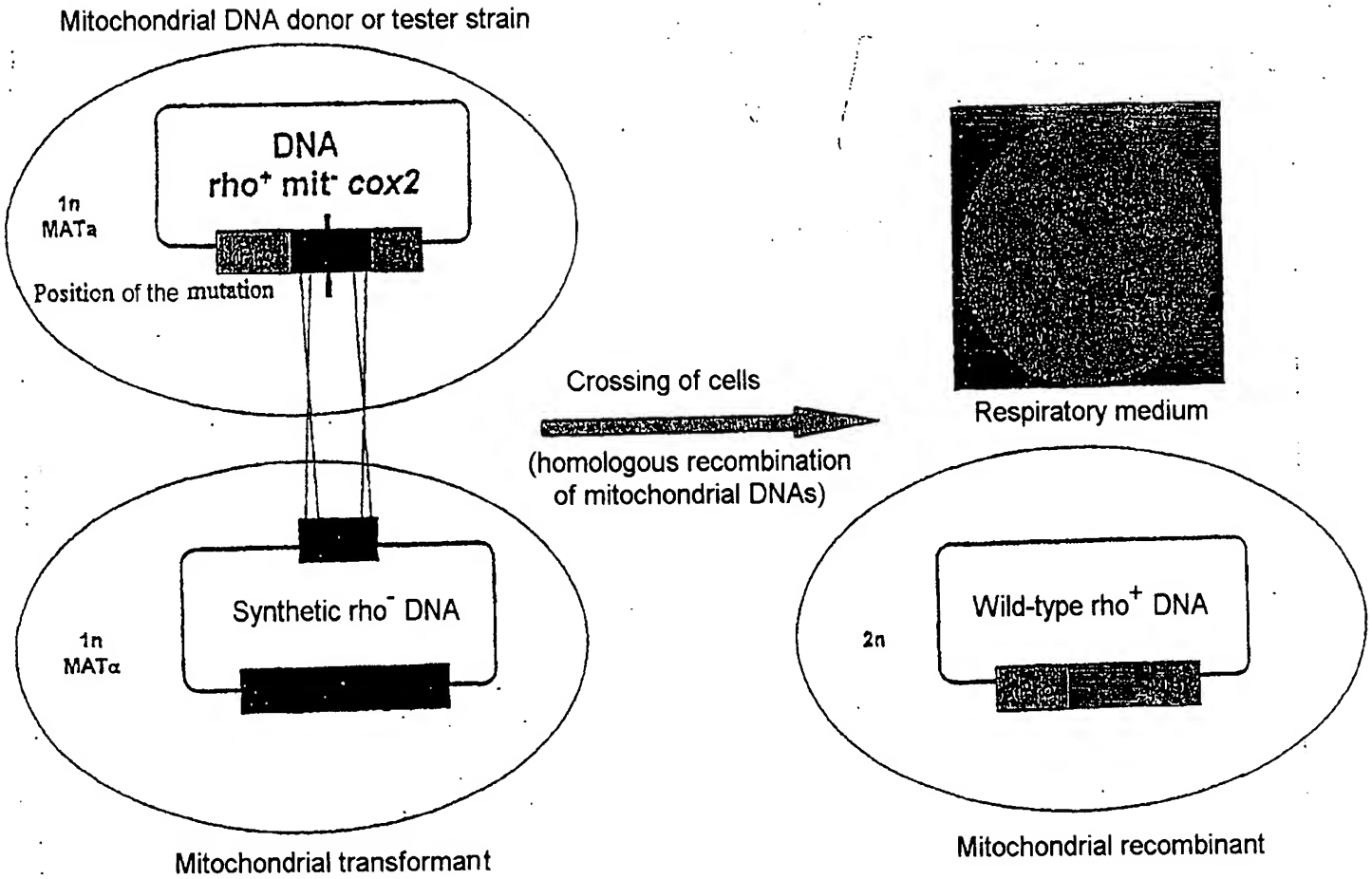
Mitochondrial DNA donor or tester strain
DNA
rho+ mit- cox2
1n
MATa
Position of the mutation
Crossing of cells
(homologous recombination
of mitochondrial DNAs)
Respiratory medium
Synthetic rho- DNA
1n
MATα
Wild-type rho+ DNA
2n
Mitochondrial transformant
Mitochondrial recombinant

FIGURE 3

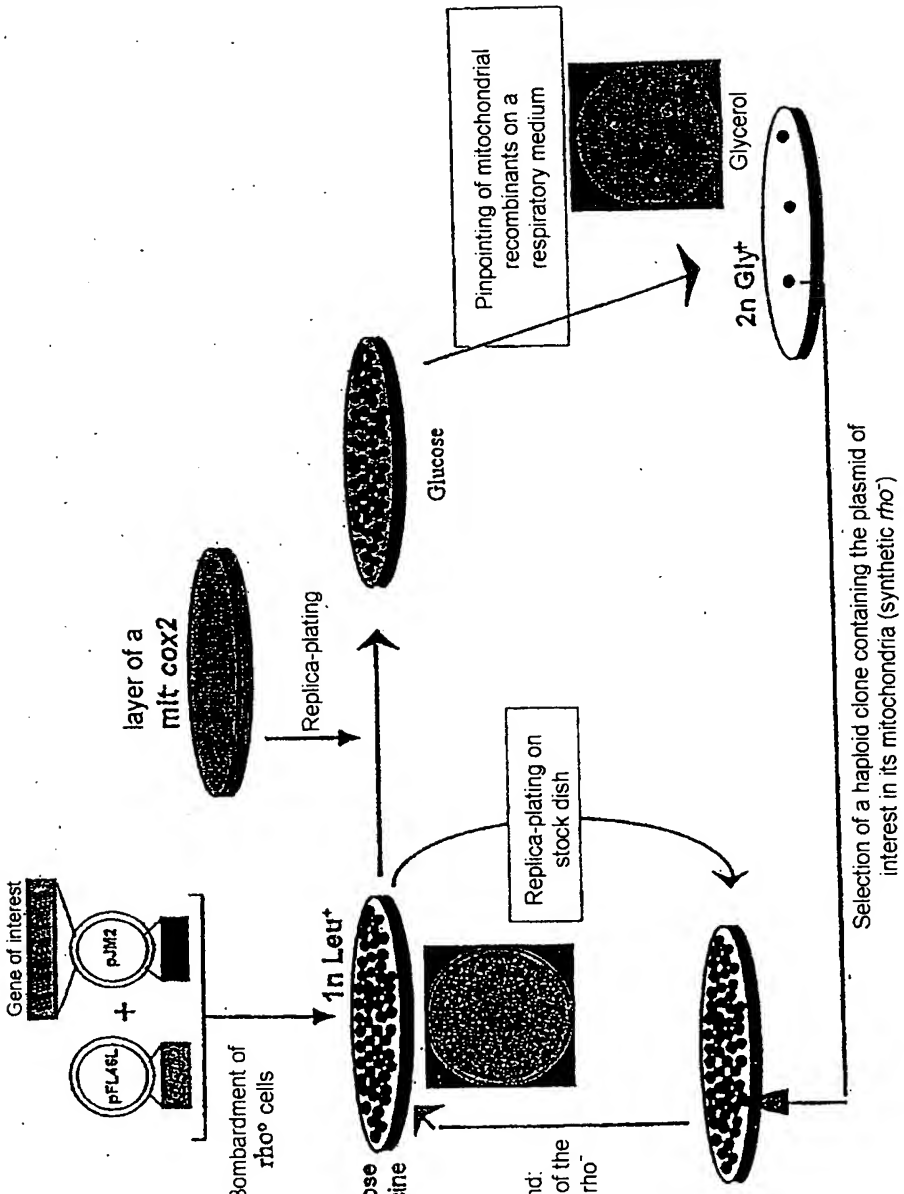
Gene of interest
pJM2
pFL46L
Bombardment of rho° cells
1n Leu+
Glucose - leucine
2nd round: purification of the synthetic rho-
Replica-plating on stock dish
layer of a mit- cox2
Replica-plating
Glucose
Pinpointing of mitochondrial recombinants on a respiratory medium
Glycerol
2n Gly+
Selection of a haploid clone containing the plasmid of interest in its mitochondria (synthetic rho-)

FIGURE 4